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Comparison of mRNA ratios of an non-DNA binding estrogen receptor (ER_α) isoform, missing exon 3 ($ER_\alpha\Delta3$), to the full length ER_α, in breast cancer, cancer cell lines and normal mammary epithelial cells and fibroblasts, revealed a 29 fold reduction of this ratio in cancer cells ($p < 0.001$). This suggested a link between $ER_\alpha\Delta3$ reduction and breast carcinogenesis. To test directly its effect on breast cancer cells, stable clones of MCF-7 cells expressing ectopic $ER_\alpha\Delta3$ protein at levels not exceeding those of physiological ER_α, were generated. In vector transfected controls the $ER_\alpha\Delta3$-mRNA and protein were less than 10% of total ER_α, while in the $ER_\alpha\Delta3$-expressing clones, $ER_\alpha\Delta3$-mRNA and protein represented approximately 50% of the total ER_α. Estrogen (E2) stimulation of pS2-mRNA was inhibited by more than 90% in all $ER_\alpha\Delta3$ -MCF-7 clones, as compared with the pMV7 controls. In presence of $1x\ 10^{-8}$ M E2, compared to control cells, the $ER_\alpha\Delta3$-expressing cells were density arrested at 50%, and their invasiveness *in vivo* was reduced by up to 79%. As expected, estrogen stimulated anchorage independent growth of both the control pMV7 and the parental MCF-7 cells, but reduced it to below baseline levels in $ER_\alpha\Delta3$ clones. The relative lack of response of pS2 gene expression to E2, and the blocking of anchorage-independent growth by E2 suggest that, aside from the dominant negative effect, E2 may activate an additional, $ER_\alpha\Delta3$-dependent inhibitory pathway. The drastic reduction of $ER_\alpha\Delta3$ to ER_α ratio in breast cancer, combined with the fact that $ER_\alpha\ \Delta3$ reverts the stimulation of the phenotypic tumor properties by E2 to suppression, suggests that an altered splice regulation of ER_α -mRNA may be a component of breast carcinogenesis.

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Irina Eckberg

PI - Signature Date

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INTRODUCTION

Estrogen, a key regulator of normal breast growth and differentiation, has been shown to promote both cancer cell proliferation and invasion (1,2). Although the estrogen receptor (ER_α) is the major mediator of estrogen action, the precise mechanism by which ER_α contributes to altered estrogen response in cancer remains unclear.

Estrogen receptor (ER_α) is one of many transcriptional regulatory proteins of the steroid receptor family that act principally as ligand-activated DNA-binding dimers (3). ER_α has distinct functional domains, including two transcriptional activating regions (the NH-terminal AF1 and the COOH-terminal, ligand-dependent, AF2), an internal zinc finger, DNA-binding domain, dimerization regions, and several nuclear localization sequences (4) (Fig.1a). Like other ligand-activated transcriptional regulators (5), ER is not a single protein, but rather a set of proteins coded by two genes giving rise to ER_α and ER_β (6) as well as isoforms generated by alternative splicing (exon skipping) of a single pre-mRNA. Since alternatively spliced ER_α-mRNAs were first noted in breast tumors and tumor cell lines, before their normal counterparts were thoroughly examined, it was proposed that overexpression of aberrant ER_α isoforms is characteristic of breast cancer (7-23). One ER_α isoform ($ER_\alpha\Delta3$), missing exon 3, which encodes the second zinc finger of the DNA-binding domain, was shown to be an in-frame deletion which in an in vitro translation reaction yielded a protein of 61.2kDa (20). This protein was unable to form specific complexes with ERE or transactivate an ERE-reporter plasmid in transient transfection assays (20). In addition, $ER_\alpha\Delta3$ was capable of inhibiting ER_α function; its inhibition of ER_α binding to ERE followed the expected pattern for a dominant negative effect, while the inhibition of transactivating activity required a large excess of $ER_\alpha\Delta3$ (20). The importance of dominant negative receptors in controlling cellular responses to agonists and antagonists has been underscored by several recent studies of the steroid receptor family (25-28). Given that $ER_\alpha\Delta3$ is a naturally occurring form of ER_α, as such, if expressed at high relative levels to full length ER_α, it may have a profound effect on several estrogen dependent functions. For example, $ER_\alpha\Delta3$ expression in normal breast tissue may provide a means of regulating the magnitude of estrogen responses, and a relative loss of $ER_\alpha\Delta3$ expression in breast tumor tissue may lead to unchecked estrogen stimulation. Alternatively, a rise in $ER_\alpha\Delta3$ expression during breast carcinogenesis may facilitate the disabling of the normal differentiation-inducing function of estrogen. Finally, the isoform may represent such a minor component that it would not influence estrogen mediated pathways in either normal or malignant breast tissue.

To distinguish between these possibilities we have compared the relative levels of $ER_\alpha\Delta3$ and ER_α expression in breast cancers, and cell lines, and in luminal and basal epithelium and fibroblasts purified from reduction mammoplasty specimens. This comparison, and the subsequent analysis of breast cancer cells expressing ectopic $ER_\alpha\Delta3$, yielded strong support for the hypothesis that $ER_\alpha\Delta3$ causes a profound change in cell response to E2 and that the relative loss of $ER_\alpha\Delta3$ may be important in carcinogenesis.

METHODS

Estrogen receptor RT-PCR. Total RNA was extracted using RNAzol B reagent (Biotecx Laboratories, Houston, TX), and 1 μg was reverse-transcribed using Superscript reverse transcriptase (Gibco BRL, Githerburg, MD) and a primer specific to exon 4 (5'-GGAGACATGAGAGCTGCCAAC-3') of ER_α. This Exon 4 primer and a primer specific to exon 2 (5'-CCGCAAATGCTACGAAGTGG-3') were used to amplify ER_α-cDNA in a 25 cycle reaction of 1 min. each at 95°, 60° and 72°. PCR products were fractionated on a 2% agarose gel, Southern blotted onto Hybond nylon membrane (Amersham, Arlington Heights, IL), and probed using either a ^{32}P end-labeled internal exon 4 probe (5'-GAATGTTGAAACACACAAGCGCC-3'), detecting full length ER_α and $ER_\alpha\Delta3$ or an exon 3 specific probe (5'-CCGCAAATGCTACGAAGTGG-3') detecting full length ER_α only. Quantitation was performed using the phosphoimager ImageQuant program.

Preparation of $ER_\alpha\Delta3$ ($ER_\alpha\Delta3$/pMV7) expression vector. A partial ER_α-cDNA fragment, containing exons 1, 2 and 4, but missing exon 3 (21), (a gift of Dr. R. Miksicek, SUNY, Stony Brook, NY) was used to replace exon 1 - 4 in a similarly digested HEGO vector (a gift of Dr. P. Chambon, Strasbourg, France). The resulting $ER_\alpha\Delta3$ coding sequence was purified and ligated into a retroviral expression vector, pMV7 (32) under the MuLV promoter. This vector also contains the neomycin resistance gene. The "empty" pMV7 plasmid served as a vector control. Both vectors were used to transform DH5a bacteria and the DNAs purified using Wizard Maxi-Prep kit (Promega, Madison, WI). To prepare $ER_\alpha\Delta3$-coding retrovirus for infection, $ER_\alpha\Delta3$/pMV7 DNA was transfected into an amphotropic packaging cell line Ψ-CRIP, selected with G418, the virus was collected, pooled and used for infection.

Maintenance of control and $ER_\alpha\Delta3$ clonal cells. MCF-7 cells were maintained in RPMI-1640 medium supplemented with insulin (5 μg/ml), penicillin (50U/ml), streptomycin (50 μg/ml), and 10% fetal bovine serum (FBS) (JRH, Lenexa, KS). All transfected cells were maintained in selection medium with 500 μg/ml G418. For growth of $ER_\alpha\Delta3$ clones, 10% charcoal stripped FBS (csFBS) was used, unless otherwise noted.

Separation of epithelial and stromal cells. Normal reduction mammoplasty specimens or breast cancer samples were obtained from the Pathology Department, Mount Sinai Medical Center. Epithelial organoids were separated from stroma by mincing and incubating the tissue overnight in hyaluronidase/collagenase as described (53). Tumor tissue was minced and

incubated in collagenase for 2 hrs at 37 ^{0}C. The organoids were collected by filtering the digest through a 400 mesh sieve, and trypsinized into single cell suspensions. The filtered single cells were plated and enriched for fibroblasts by differential trypsinization. Tumor digest was plated without filtration and the cultures enriched for fibroblasts by differential trypsinization.

Separation of basal and luminal epithelium. Basal epithelial cells were positively selected from the filtered digest using a monoclonal antibody to the common acute lymphoblastic leukemia (CALLA) antigen (DAKO, Carpinteria CA) and Dynabeads (Dynal, Norway) coated with goat anti-mouse IgG (a 10:1 bead:cell ratio) essentially as described (54). These basal epithelia enriched cells were cultured in mammary epithelial cell growth medium (MEGM; Clonetics, San Diego CA) with 5 μg/ml transferrin and 10 μM isoproterenol. The CALLA negative fraction, containing the luminal cells, was densely seeded onto collagen I-coated dishes in MEGM. After a week in culture, RNAs were extracted and cell purity determined by Northern blot analysis of cytokeratin expression (K8-luminal and K5-basal). Cell preparations with K8 to K5 ratios of 10 to 1 or 1 to 10, were defined as luminal or basal cells, respectively. Some epithelial cell preparations, not purified further, were used and designated "unselected". RNAs were extracted from these cell types and used in RT-PCR assay for $ER_\alpha\Delta3$ and ER_α analysis.

Generation of MCF-7 cells expressing $ER_\alpha\Delta3$ (Transfection/Infection). Three μg of $ER_\alpha\Delta3$/pMV7 or pMV7 DNA was transfected with Lipofectin into MCF-7 cell, as per manufacturer's recommendation. For retroviral infection (44) 2 ml of growth medium containing the virus and 8μg/ml of polybrene were added to semi-confluent MCF-7 cells, the cells were rocked for 2 hrs at 37^0 C, the inoculum removed, cell incubated in medium with serum for 48 hrs and transferred to medium with G418 (selection medium). Infected and transfected cells were maintained in G418-containing medium for 1-2 months prior to clone isolation.

Immunoprecipitation and western blotting. Total cell protein was prepared by 4 freeze/thaw cycles in a high salt lysis buffer (0.4M NaCl,10% Glycerol, 1mM DTT, 100mM Tris, 10mM EDTA, 50 μg/ml Leupeptin, 50 μg/ml Aprotinin, 10 μg/ml Pepstatin). ER_α and $ER_\alpha\Delta3$ were immunoprecipitated with a rabbit anti ER_α antibody (Zymed, San Francisco, CA), and protein G agarose (Boerhinger Manheim, Indianapolis, IN) from 400 μg of total protein diluted with lysis buffer without NaCl for a final NaCl concentration of 0.2M. Immunoprecipitated material was resuspended in 50μl of loading buffer, and electrophoresed on an 11.5% SDS/PAGE gel for 8 to 10 hrs at 200 Volts. Protein was transferred onto nitrocellulose membrane (Amersham), blocked overnight with 5% non-fat milk, washed in TBST/1% non-fat milk, and western blotted with H226 (0.7mg/ml) rat anti ER_α primary antibody (1:100 dilution) overnight at 4^{0}C, and incubated with an HRP-conjugated goat anti-rat secondary antibody (1 : 10,000 dilution) (Sigma, St.Louis, MO) for 1hr at room temperature. Chemiluminescence (ECL Kit, Amersham) detected bands were quantitated by densitometry.

Phosphatase treatment of protein extract from pMV7 and $ER_\alpha\Delta3$ clone. Total ER_α was immunoprecipitated from 2 mg of protein from pMV7pool and $ER_\alpha\Delta3$ clonal cells, using rabbit polyclonal anti-ER_α antibody (Zymed). Immunoprecipitated material was split into two equal aliquots, resuspended in 25 μl 1X phosphatase buffer (Boerhinger Mannheim, 10 x phosphatase buffer: 0.5 M Tris-Hcl pH 8.5, 1 mM EDTA), containing a 2X protease cocktail (100 μg/ml Leupeptin, 200 μg/ml Bacitracin, 100 μg/ml Aprotinin, 20 μg/ml Pepstatin). One aliquot of each of pMV7pool and $ER_\alpha\Delta3$-3, was treated with 3 Units of calf intestinal phosphatase (CIP) (Boehringer Mannheim), and along with the mock treated aliquots, were incubated for 30min. at 30^{0}C. The reaction was terminated by the addition of 25 μl of 2X loading buffer and heating to 95^{0}C for 3 min. Western blot analysis was performed as described above.

Expression of pS2. pMV7pool and $ER_\alpha\Delta3$-1, 2, 3, and 4 clonal cells (1x10^6) were grown for 3 days in 100mm tissue culture dishes in the presence of FBS, and subsequently treated with either ICI 164,384 (1x10-7) or two concentrations of E2 (1x10^{-8} M and 1x10^{-10} M) for 2 days. pS2 expression was assessed by northern blotting 20 μg of total RNA, hybridized with random primed pS2 and GAPDH (as a loading control) cDNA probes. pS2-mRNA level was determined by Densitometric analysis.

Saturation density. pMV7pool and $ER_\alpha\Delta3$ clonal cells (4x10^6) were plated in 100 mm tissue culture dishes in the presence of FBS and 1x10^{-8}M Estradiol (E2) (Sigma). Cells were maintained for four days beyond visual confluence, with a medium change every 2 days, trypsinized and counted. Mean and standard deviation were calculated from four independent experiments.

Growth in soft agar. A two layer low melt agarose (Seaplaque) system was used to assess anchorage independent growth of pMV7pool and $ER_\alpha\Delta3$ clonal cells. A 1% lower layer and an 0.4% upper layer of agarose, prepared in DMEM medium supplemented with insulin (5 μg/ml), penicillin (50 U/ml), streptomycin (50 μg/ml), and 10% FBS (+/-E2 1x10 $^{-8}$M or +/- Tamoxifene 1 x 10 $^{-6}$ M) inoculated into 60mm gridded plates. Cells (2 x10^3 cells/ml), distributed in the upper layer, were allowed to grow for 2 weeks and colony formation in the three conditions was scored. The effect of E2 and Tamoxifene was determined by comparison to cloning efficiency in FBS.

Chorioallantoic membrane invasion. Invasion was assayed as previously described (39). $ER_\alpha\Delta3$ clones or pMV7pool cells were grown in the presence of selection medium supplemented with 10% FBS and estradiol (1x10^{-8}M) for 48 hrs.. Cells

were trypsinized, counted, allowed to attach overnight in the same medium ($4x10^6$ cells per 100-mm dish), and labelled with 0.2 μCi/ml of ^{125}IUdR for 24 hrs. (specific activity of 0.1 - 0.2 cpm/cell). An artificial air chamber above the CAM of a 10 day old embryo was created, and the CAM was allowed to reseal for 22 hrs and the labelled cells ($3x10^5$ per CAM) were inoculated onto the CAM. Following a 24 hr. incubation, CAMs were washed with PBS, excised, incubated for 20 min in trypsin-EDTA (0.05% trypsin, 1mM EDTA), to remove surface attached tumor cells, and rinsed with PBS. The radioactivity remaining in CAMs after the trypsin-EDTA incubation and the PBS wash, expressed as percent of total radioactivity (associated with CAMs before trypsinization and present in washes), represent the proportion of cells that invaded. The Mann-Whitney U test was used for the statistical analysis.

RESULTS

Comparison of ER_α and $ER_\alpha\Delta3$ expression in cancer and normal breast cells.

RNA extracted from aliquots of 33 breast cancers was analyzed for ER_α and $ER_\alpha\Delta3$ expression using a semi-quantitative RT-PCR assay capable of distinguishing between mRNA encoding the full length and the $ER_\alpha\Delta3$ forms of the receptor. The two forms of ER_α were detected by Southern blot analysis of ER_α-cDNA amplified with primers within exons 2 and 4, using internal probes hybridizing either with exon 4, to detect both forms of the receptor, or within exon 3 to detect only the full length ER_α. The median ratio of $ER_\alpha\Delta3$ to ER_α expression in these tumors was 0.12 (range of 0.03 to 0.47) (Fig.1b, upper panel and 1d, group 1). Analysis of 9 cancer cell lines, (7 of which were ER_α positive) confirmed the low relative levels of $ER_\alpha\Delta3$ in pure populations of cancer cells (median ratio of 0.1, range 0.06 to 0.3, Fig. 1b, lower panel and 1d, group 2). A similar tests of epithelial cells isolated from 10 reduction mammoplasties, which included purified populations of luminal and basal cells, unselected epithelial cells and one immortalized, myoepithelial mammary cell line (Hs 578Bst), yielded a median ratio of $ER_\alpha\Delta3$ to ER_α of 3.4 (range 0.4 to 9.8), which is approximately 29 fold greater than the ratio found in breast cancer (Fig. 1c and 1d, group 3). Interestingly, breast fibroblasts were also found to have high $ER_\alpha\Delta3$ to ER_α ratios (median 2.4, range 1.5 to 4.5), regardless of their source (reduction mammoplasty, n= 4 or breast cancer, n=2). (Fig. 1d, group 4). The median ratio of $ER_\alpha\Delta3$ to ER_α in three purified luminal epithelial cell populations, as identified by the predominance of cytokeratin 8 expression (K8/K5 ratio of 10:1), (Fig.1d, lane 4, encircled crosses) was 0.42 (range 0.36 to 0.53) a value almost 4 fold greater than the median ratio in primary cancer tissue or cancer cell lines. This is significant since cytokeratin K8, normally present in luminal epithelium, have been shown to be expressed in a large proportion of breast cancers (29, 30) suggesting that these cells may be the target of oncogenic transformation in breast. In basal cells and unselected epithelial cells, most of which contained predominantly basal cells, (Fig.1d, lane 4) the median $ER_\alpha\Delta3$ to ER_α ratio was even greater (median 4.0; range 0.55 - 9.8). This high ratio may explain the low in vivo proliferation rate of basal epithelial cells (24).

Stratification of patients according to their menopausal status or tumor stage (defined by tumor size, (< 1.5 cm or > 1.5 cm), or presence or absence of lymph node involvement) did not identify significant difference in the $ER_\alpha\Delta3$ to ER_α ratios. The exception was a group of tumors (6/33) with ER_α levels, defined by routine ligand binding as lower than 5 fmole/mg protein, and deemed ER_α negative, in which the median ratio (0.05) was significantly different ($p < 0.001$) from the median ratio (0.12) of all tumors.

Cumulatively these results, showing that the ratio of $ER_\alpha\Delta3$ to full length ER_α is substantially reduced in all breast cancer cell lines and in breast cancers, even when tumors are smaller than 1.5 cm and have not spread to the lymph nodes, suggest that a loss of the $ER_\alpha\Delta3$ isoform may be associated with an early event in carcinogenesis.

Transfection and isolation of MCF-7 cells expressing $ER_\alpha\Delta3$; characterization of the native and transgenic protein.

The above findings suggested that restoring $ER_\alpha\Delta3$ in cancer cells to normal relative levels may result in attenuating their transformed phenotype. To test this, $ER_\alpha\Delta3$-cDNA was subcloned into a pMV7 vector (32). The $ER_\alpha\Delta3$/pMV7 construct was tested by transiently transfecting COS cells, negative for ER_α, and showing (Fig. 2) that transfected cells express and properly localize $ER_\alpha\Delta3$ to the nucleus, and that the $ER_\alpha\Delta3$ protein reacts with a well characterized rat anti-estrogen receptor antibody (H226) (33).

Stable clonal lines of MCF-7 cells were then selected from cultures transfected (or infected) with either the $ER_\alpha\Delta3$ coding constructs, or the PMV7 vector alone for negative control, and analyzed both for $ER_\alpha\Delta3$-mRNA, by RT-PCR (Fig. 3a), and protein expression, by immunoprecipitation and western blotting, (Fig. 3b). Attempts to generate specific $ER_\alpha\Delta3$ antibodies that recognize the exon 2/exon 4 splice junction were unsuccessful. Therefore, the $ER_\alpha\Delta3$ protein was identified on the basis of its reactivity with two antibodies recognizing different N-terminal epitopes of ER_α, its faster mobility than ER_α on SDS-PAGE, and the correlation of its expression with that of $ER_\alpha\Delta3$-mRNA.

As expected, (Fig. 3a, lane 5) control pMV7 cells expressed predominantly full length ER_α-mRNA and a small amount of $ER_\alpha\Delta3$, similar to that observed in the parental MCF-7 cell line (Fig. 1b, lane 8). In contrast, all four $ER_\alpha\Delta3$/pMV7 clones had a predominance of $ER_\alpha\Delta3$-mRNA (Fig. 3a lanes 1 - 4), indicating that the transgene mRNA was efficiently expressed in these cells.

Extracts of the individual clones shown in Fig. 3b were subjected to immunoprecipitation with a polyclonal rabbit anti-ER_α antibody followed by western blotting with the H226 antibody, recognizing the amino-terminus of ER_α. In addition to the 65 kDa band, representing the full length ER_α protein, all $ER_\alpha\Delta3$ clones also contained a prominent 61 kDa band, which corresponded to the predicted molecular weight of the $ER_\alpha\Delta3$ protein. Expression of the $ER_\alpha\Delta3$ protein in these clones ranged from approximately 40-70% of total ER_α (a relative ratio of $ER_\alpha\Delta3$ to ER_α of 0.7 - 2.3), comparable to that observed in the normal mammary epithelium. In parental MCF-7 extracts, a faint band (~5% of the total ER_α), co-migrating with the $ER_\alpha\Delta3$ form, could be detected only when excess protein was loaded onto the gel (Fig. 4, lane 2). This, and the correspondence between the low intensity of the $ER_\alpha\Delta3$-mRNA band and the 61 kDa protein band (Fig. 1b, lane 8; Fig.3, lane 5; Fig.4, lane 2, respectively) suggest that both pMV7-carrying and the parental MCF-7 cells produce small amounts of the $ER_\alpha\Delta3$ mRNA and protein. The identity of the 61 kDa band as $ER_\alpha\Delta3$, and not as an under-phosphorylated form of full length ER_α, was confirmed in a de-phosphorylation experiment. Total immunoprecipitated ER_α from pMV7 or $ER_\alpha\Delta3$ cells was mixed with protease inhibitors, one aliquot of each was dephosphorylated by incubation with calf intestinal phosphatase (CIP), the other incubated under identical conditions but without CIP. Analysis of products by western blotting showed that without CIP, ER_α from both pMV7 and $ER_\alpha\Delta3$ cells produced a co-migrating doublet of bands, the upper corresponding to full length ER_α and the lower to $ER_\alpha\Delta3$ protein (compare lanes 2 and 3 of Fig. 4). CIP treatment shifted the migration coefficient of both bands in the vector control cells as well as the $ER_\alpha\Delta3$ clone to new positions, once more as a co-migrating doublet (Fig. 4, lane 1 and 4). No lower bands or smear were detected, indicating that proteolysis during CIP incubation was effectively blocked by the protease-inhibitors cocktail. Co-migration of the lower molecular weight protein from pMV7 cells with that of the $ER_\alpha\Delta3$ protein, both before and after de-phosphorylation, strongly suggests the presence of endogenously produced $ER_\alpha\Delta3$ protein. Similar results were obtained using the ER positive Ishikawa cells, an endometrial carcinoma cell line (data not shown).

$ER_\alpha\Delta3$ expression suppresses estrogen stimulated gene expression:

$ER_\alpha\Delta3$ has been shown to interfere with ER_α binding to its specific DNA response element *in vitro*, as well as with E2 induced transcription of an ERE-CAT reporter in transient transfection of COS cells *in vivo*. These studies suggested that $ER_\alpha\Delta3$ functions as a dominant negative receptor to inhibit ER_α regulation of gene expression through its cognate DNA response element. In order to determine whether the $ER_\alpha\Delta3$ expressed in MCF-7 cells can interfere with estrogen induction of an endogenous gene, the expression of pS2, a gene with several imperfect ERE's in its promoter, was assessed. pMV7 control and $ER_\alpha\Delta3$ clone cells were incubated either with the pure anti-estrogen, ICI 164,384 ($1x10^{-7}$ M), to establish the baseline of pS2 expression, or with E2 ($1x10^{-8}$ M and $1x10^{-10}$ M). Total RNA was prepared and analyzed by Northern blot to determine pS2 expression. (GAPDH mRNA was used as a loading control). While E2 treatment of controls induced a 25 fold increase in pS2-mRNA (compare lane 1 with lanes 2 and 3 in Fig.5aand Fig.5b), in $ER_\alpha\Delta3$-expressing cells pS2-mRNA was stimulated merely 2 fold (compare lane 4 with lanes 5 and 6 in Fig.5a and Fig. 5b). In all additional $ER_\alpha\Delta3$ clones tested (a total of 4) , E2 induction of pS2-mRNA ranged from only 3-9% of that observed in the pMV7 control (results not shown). These results confirm that the $ER_\alpha\Delta3$ s interferes with the ER_α regulated gene expression *in vivo.*

$ER_\alpha\Delta3$ expression alters growth properties of MCF-7 cells:

During the initial selection in medium with FBS, the $ER_\alpha\Delta3$/pMV7 transfected clones grew much slower than the parental cells or the vector-transfected clones; the $ER_\alpha\Delta3$/pMV7 cells divided once every 6 days while the pMV7 clones divided every 4 days. Microscopic observation of these cells also suggested that the $ER_\alpha\Delta3$/pMV7 cells stopped dividing at lower confluence level. To further evaluate this difference, $ER_\alpha\Delta3$ clones and pMV7 controls were plated at 50% confluence in medium containing FBS with E2, maintained for 4 days beyond visual confluence and counted. Results in Fig. 6 show that $ER_\alpha\Delta3$ clones reached a plateau in cell density at cell number that was only 50% of the controls, perhaps indicating that cells expressing $ER_\alpha\Delta3$ are more sensitive to signals of contact inhibition. This was the first suggestion that the expression of $ER_\alpha\Delta3$ shifts the transformed phenotype of breast cancer cells toward behavior expected of normal cells.

Shifting $ER_\alpha\Delta3$-expressing cells into estrogen depleted, charcoal stripped FBS, (csFBS) stimulated their growth relative to their growth in FBS. This effect on growth was accompanied by a change in the $ER_\alpha\Delta3$ to ER_α protein ratio. Western blot detection of estrogen receptors immunoprecipitated from an equal amount of protein of $ER_\alpha\Delta3$ clone 2 cells, grown either in the presence of csFBS or E2 supplemented FBS showed that there was more overall receptor protein in cells grown in medium with csFBS (Fig. 7), and that the gain was predominantly in the full length receptor, thus increasing the ER_α to $ER_\alpha\Delta3$ protein ratio. (Similar results were obtained with $ER_\alpha\Delta3$ clone 1, data not shown). These data indicate that when $ER_\alpha\Delta3$ predominates (as in FBS-E2 containing medium) cell growth is retarded. (Such contrasting requirements for optimal growth of the controls and $ER_\alpha\Delta3$-expressing cells preclude meaningful comparisons of growth rates under the same conditions). To shift the $ER_\alpha\Delta3$ to ER_α ratio in favor of the transgenic protein, all further experiments were carried out on cells grown in medium with FBS and estradiol (although for daily cell maintenance the clones were kept in medium supplemented with csFBS).

$ER_\alpha\Delta3$ attenuates the transformed phenotype of MCF-7 cells.

An in vitro property of tumor cells that is thought to predict their in vivo tumorigenicity is their ability for anchorage-independent growth. We examined the consequence of $ER_\alpha\Delta3$ expression on the anchorage-independent growth of MCF-7 cells (Fig. 8). As shown by others, estrogen stimulated the ability of parental MCF-7 cells (and of the pMV7 control cells) to form colonies in soft agar. In contrast, hormone treatment drastically reduced the ability of $ER_\alpha\Delta3$ expressing clones to form colonies in agar, even below the baseline level (Fig. 8). The magnitude of this reduction was similar to that obtained with an anti-estrogen, tamoxifene (Fig. 8). Accordingly, these data suggest that, in vivo, $ER_\alpha\Delta3$ may reverse the tumorigenic phenotype of breast cancer cells through an as yet undetermined mechanism.

To assess the effect of $ER_\alpha\Delta3$ expression on the ability of MCF-7 cells to invade host tissue, which is linked to protease production known to be under the control of E2 in these cells (34-38), we inoculated chick embryo chorioallantoic membrane (CAMs), in vivo, with a pMV7 clone or $ER_\alpha\Delta3$ expressing clones 1,2,3,4 grown in the presence of E2 and metabolically labeled with ^{125}IUdR for 24 hrs, and measured their invasive ability 24 hrs later by a previously described method (39). We determined that, compared with the parental MCF-7 cells or pMV7 vector control cells, the ability of $ER_\alpha\Delta3$-expressing clones to invade CAM was reduced by 52-79% (Fig. 9).

DISCUSSION

We determined that a non-DNA binding ER_α isoform ($ER_\alpha\Delta3$) is expressed in normal breast epithelial cells with a median ratio of $ER_\alpha\Delta3$ to ER_α of 3.4, with a subset of purified luminal epithelial cells having a median ratio of $ER_\alpha\Delta3$ to ER_α of 0.4. In contrast, the median $ER_\alpha\Delta3$ to ER_α ratio in breast tumors and tumor cell lines is only 0.1, indicating a substantial under-representation of $ER_\alpha\Delta3$ in cancer cells.

It is a paradox that the same hormone, estrogen, exerts the tightly controlled effects on growth and differentiation of normal breast cells during puberty, and on their cyclical proliferation in an adult non-pregnant female, while also acting as a potent mitogen in breast cancer during its uncontrolled growth and invasion (1,2). This dichotomy suggests that, during oncogenic transformation, mammary epithelial cells may undergo signaling pathway changes leading to aberrant or inappropriate estrogenic responses. The evidence presented in the current study is the first demonstration that a selective loss of $ER_\alpha\Delta3$ may contribute to the phenotypic changes of cancer. The observation showing that small tumors, or tumors that have not spread to the lymph nodes, have ratios of $ER_\alpha\Delta3$ to ER_α as low as the more advanced tumors, suggests that the loss of $ER_\alpha\Delta3$ may be an early event in carcinogenesis. (However, the finding of significantly lower ratios in tumors with $ER_\alpha < 5$ fmole/mg, considered more aggressive, hints that a further drop of $ER_\alpha\Delta3$ may be associated with disease progression.) It is also worth noting that, in spite of the fact that these tumors and the normal breast cells have equally low levels of the receptor, they have contrasting ratios of $ER_\alpha\Delta3$ to ER_α, indicating that the high relative level of $ER_\alpha\Delta3$ in normal cells is not the consequence of their overall low receptor level.

The high relative expression of $ER_\alpha\Delta3$ in normal breast epithelium and fibroblasts, may provide them with a mechanism to regulate and limit the magnitude of responses to estrogen. It can be argued then, that to attain maximum estrogen stimulation of growth and invasive potential during carcinogenesis, breast cells need to be released from the effects of $ER_\alpha\Delta3$. Accordingly we demonstrated that a selective loss of this receptor occurs in breast tumors and breast cancer cell lines and that the re-introduction of physiologically relevant levels of $ER_\alpha\Delta3$ into breast cancer cells attenuates the mitogenic action of estrogen and reverses several features that distinguish transformed from normal cells.

Most studies of ER_α in normal human mammary tissue have used relatively insensitive immunohistochemical or biochemical techniques. Consequently, only a subset of luminal epithelial cells, and no other cells in normal breast tissue, were considered receptor positive (40). Our study, using RT-PCR, demonstrated ER_α expression both in luminal and basal/myopeithelial cells of the normal breast epithelium (Fig. 1c), as well as in stromal fibroblasts, (unpublished results). Moreover, mammary fibroblasts, demonstrated to be estrogen responsive (41,42) were confirmed to express ER_α protein by more sensitive immunofluorescence techniques using a strep-avidin amplification of anti-ER_α antibodies (unpublished results). Thus, several different cell types in the normal adult breast may respond directly to E2. Also, as the high $ER_\alpha\Delta3$ ratio (median 2.4) is preserved in breast cancer fibroblasts, it is likely that their presence in cancer tissue may contribute to some degree to the difference in $ER_\alpha\Delta3$ to ER_α ratios found in tumors.

The current study has identified the $ER_\alpha\Delta3$ protein in cell lines expressing the $ER_\alpha\Delta3$ transgene as well as in parental MCF-7 cells and Ishikawa cells; in all cases the $ER_\alpha\Delta3$ to ER_α protein ratios were similar to the $ER_\alpha\Delta3$ to ER_α RNA ratios (Figs. 3 and 4). The very low abundance of ER_α in normal mammary cells preclude such a direct analysis of ER_α protein in these cells. However, the finding that in cell lines ER_α-RNA ratios reflect those of the corresponding proteins, make the likelihood of such correspondence in normal cells highly plausible.

The relevance of our findings is further underscored by the demonstration of an autoregulatory loop in the clones with re-expressed $ER_\alpha\Delta3$. In these cells exposure to estrogen can shift the complement of estrogen receptors from mostly ER_α to predominantly $ER_\alpha\Delta3$ (Fig. 7). This is achieved by a more pronounced down-modulation of ER_α than of $ER_\alpha\Delta3$ and, as shown previously (43, 44), may occur via several mechanisms, including mRNA and protein stability. If a similar

mechanism of auto-regulation exists in endogenous tissue, then during periods of peak estrogen availability, a rise in the $ER_\alpha\Delta3$ to ER_α protein ratio may protect breast tissue from over-stimulation. Thus, oncogenic transformation of breast cancer cells, resulting in a selective reduction in $ER_\alpha\Delta3$ expression, would lead to a disruption of this response, promote un-checked estrogen action, and establish permissive conditions for further carcinogenic events.

The re-establishment of a less tumorigenic phenotype in the $ER_\alpha\Delta3$-transfected MCF-7 cells deserves further comment because certain of the properties, such as reduced plateau density and reduced invasion may result be the result of dominant negative inhibition by $ER_\alpha\Delta3$, while others, such as anchorage-independent growth, may be mediated via additional pathways. Since, as noted, in the $ER_\alpha\Delta3$-transfected clones, the relative level of this isoform is highest in the presence of estrogen, it is interesting that estrogen treatment of these clones causes a marked reduction of growth and, more importantly, a much lower saturation density, as is characteristic of a normal phenotype. These effects are specific to the $ER_\alpha\Delta3$ isoform, since a similar transfection of full length ER_α into either MCF-7 or T47D cells (which are also ER_α-positive), did not reduce their proliferative response to hormone (45). Although not yet examined, a testable hypothesis is that a dominant negative receptor interferes with estrogen stimulation of genes critical for growth regulation, such as cyclin D1, myc, and the fos/jun family of transcription factors (46, 47). These gene products, in turn, may reduce growth factor receptor expression, resulting in a lower saturation plateau.

The reduced invasiveness of the $ER_\alpha\Delta3$-expressing cells may be also mediated via a dominant negative effect. It is known that estrogen is necessary for MCF-7 tumor growth and metastasis in nude mice. Estrogen also stimulates the expression of several proteolytic enzymes (such as plasminogen activators, collagenase IV, cathepsin D), shown to be involved in cancer invasion (34-38). It is likely that the presence of $ER_\alpha\Delta3$ will effectively interfere with stimulation of these proteases by E2 to result in reduced invasiveness.

In contrast to the above effects, E2 not only fails to stimulate anchorage independent growth in $ER_\alpha\Delta3$ expressing cells, but inhibits it to below baseline levels, as obtained with the anti-estrogen, tamoxifene. This inhibition cannot be explained purely on the basis of a dominant negative effect, since in most of the clones tested, the ratio of $ER_\alpha\Delta3$ to ER_α is not greater than 1 to 1 and suggests the existence of an additional pathway of $ER_\alpha\Delta3$ action. This conclusion is supported by published observation showing that co-transfection of $ER_\alpha\Delta3$ and ER_α proteins, at ratios comparable to those present in our clones, produced only a 30% inhibition of estrogen dependent transactivation (20), and is further supported by our findings of almost complete abolishment of E2 stimulation of pS2-mRNA in cells expressing $ER_\alpha\Delta3$. Since the total ER_α level in the clones is either equal to, or less than, that in the parental MCF-7 cells, the observed effect could not be due to the general over-expression of ER_α protein, shown by some to lead to E2 inhibition of growth (48, 49). Although, we have not yet investigated the mechanism of the suppressive signal transduction pathway of $ER_\alpha\Delta3$, it is likely that this receptor isoform, in addition to its dominant negative action, participates in the non-classic regulation of gene expression via protein protein interaction with other transcription factors, that have been shown recently to be both, independent of ER_α binding to DNA (50), and importantly, independent of the ER_α-DNA-binding domain (51).

Thus we have demonstrated a novel function for a non-DNA binding estrogen receptor isoform in breast biology. Relative high expression of this isoform in normal mammary tissue may provide a mechanism for attenuating estrogenic effects, and its reduction in breast cancer may lead to excessive, unregulated mitogenic action of this hormone. Our results indicate that, as with tumor suppressor WT1 (52), carcinogenic events in breast can lead to alteration of splice choice pathways, but unlike suggested for other ER_α isoforms (8-12,14,15,17-19,21), rather than being elevated in cancer, the relative ratio of this isoform is diminished. Further studies of the mechanisms through which $ER_\alpha\Delta3$ exerts its effect will clarify its role in controlling E2 responsiveness in mammary cells. Identifying ways to re-direct the pathway towards enhanced expression of $ER_\alpha\Delta3$, or finding alternative means of increasing its relative ratio, may provide a novel avenue for future breast cancer therapy.

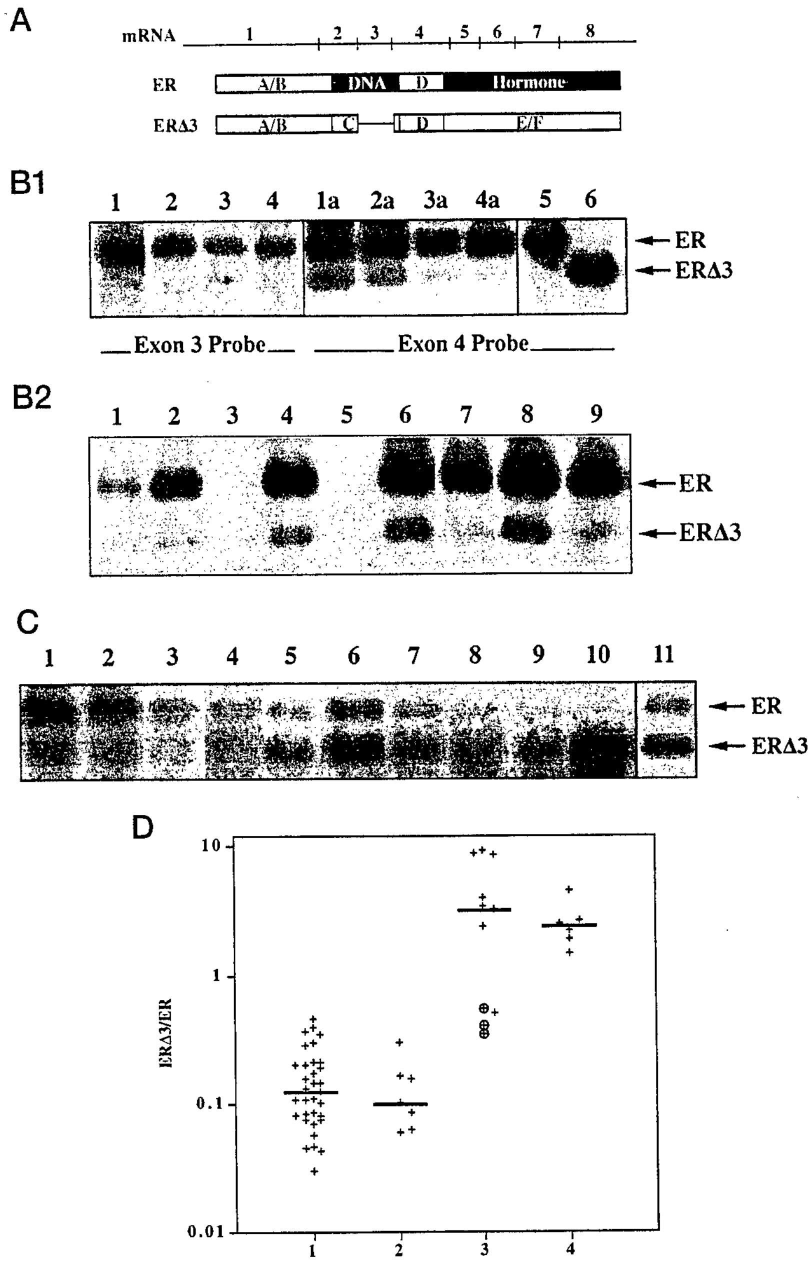

Fig. 1. Analysis of ER_α and $ER_\alpha\Delta3$ mRNA Expression in Normal Breast and Breast Cancer Tissue and Cells

A, Diagram of mRNA and protein structures of ER_α and $ER_\alpha\Delta3$. B1: Southern blots of four cDNAs, obtained by RT-PCR of mRNA of breast cancers, probed with either an exon 4 probe (to detect both ER_α and $ER_\alpha\Delta3$) lanes 1a-4a, 5, and 6, or an exon 3 probe (to detect only full-length ER_α), lanes 1–4. The examples shown represent the entire range of $ER_\alpha\Delta3$ to ER_α ratios found in breast cancers; they are 0.25, 0.10, 0.08, and 0.04 for lanes 1a, 2a, 3a, and 4a, respectively. B2: Detection with exon 4 probe only. Southern blot of ER_α-positive breast cancer cell lines: lane 1, BT 474; lane 2, MDAMB175vii; lane 4, MDAMB361; lane 6, MDAMB134vi; lane 7, T47D; lane 8, MCF-7; lane 9, ER_α-positive endometrial cancer cell line, Ishikawa. ER_α-negative breast cancer cell lines: lane 3, MDAMB231; lane 5, MDAMB461. C, Southern blot of epithelial cells from 10 reduction mammoplasties (lanes 1–10) and Hs 578Bst, a normal, immortalized myoepithelial cell line (lane 11) detected with exon 4 probe. (Lanes 1–3 represent purified luminal epithelial cell preparations; lanes 4–10 represent pools of epithelial cells with predominance of basal cells). D, $ER_\alpha\Delta3$ to ER_α RNA ratios in breast cancers (group 1), breast cancer cell lines (group 2), normal epithelium (group 3) (the pure luminal epithelium, n = 3, indicated by *circled crosses*), and fibroblasts (group 4) (isolated from reduction mammoplasty, n = 4, or breast cancer, n = 2). Each point in the scattergram represents the scanned relative intensity of $ER_\alpha\Delta3$ and ER_α bands produced by Southern blotting of cDNAs generated by RT-PCR of RNA extracted from individual tissue or cell samples. The median of $ER_\alpha\Delta3$ to ER_α ratios was 0.11 for breast cancers, 0.10 for breast cancer cell lines, 3.40 for normal epithelium, and 2.40 for fibroblasts. ANOVA analysis (using SYSTAT program, SYSTAT, Inc., Evanston, IL) of the $ER_\alpha\Delta3$ to ER ratio in the three groups, tumors, tumor cell lines, and normal epithelium, showed a significant difference ($P < 0.0001$). *Post hoc* analysis showed that the primary breast cancers were not different from the tumor cell lines ($P = 0.978$), but primary breast cancers and breast cancer cell lines were different from normal epithelium, $P < 0.0001$ and $P = 0.001$, respectively.

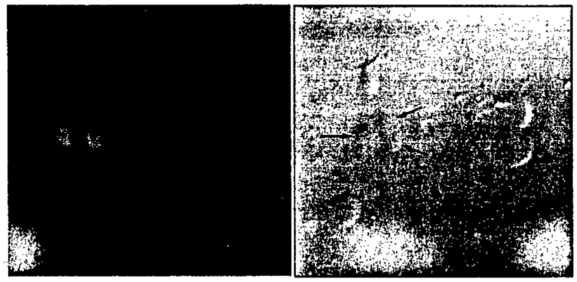

Fig. 2. Detection of Transfected $ER_\alpha\Delta3$ Protein in COS Cells

COS cells transiently transfected with $ER_\alpha\Delta3$/pMV7 vector using Lipofectin (GIBCO) were plated on coverslips 16 h after transfection, allowed to attach, fixed with 3% paraformaldehyde, and incubated overnight at 4 C with H226 antibody (35 μg/ml). Biotin-coupled anti-rat IgG secondary antibody (Sigma) and rhodamine-conjugated strepavidin (Sigma) were used for protein visualization. *Left panel*, Immunofluorescent detection of $ER_\alpha\Delta3$ in nuclei of three COS cells. *Right panel*, Nomarski optic view of the same field. Magnification ×400.

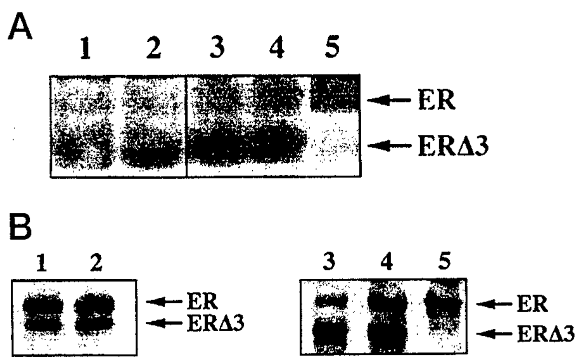

Fig. 3. Characterization of RNA and Protein from Clonal Cell Lines Obtained by Stable Transfection or Infection of MCF-7 Cells with $ER_\alpha\Delta3$/pMV7 or pMV7 Alone

A, Southern blot with exon 4 probe of RT-PCR-cDNA from $ER_\alpha\Delta3$-expressing clones and pMV7 pool vector control. Lanes 1–4 are clone $ER_\alpha\Delta3$–1, 2, 3, and 4, respectively; lane 5 is pMV7 pool vector control. B, Western blot of total ER_α immunoprecipitated from 400 μg of protein extract. *Left panel*, Experiment 1, lanes 1 and 2, clones $ER_\alpha\Delta3$–1 and 2, electrophoresis for 8 h. *Right panel*, Experiment 2, lanes 3 and 4, clones $ER_\alpha\Delta3$–3 and 4; lane 5, pMV7-pool vector control, electrophoresis for 10 h. *Arrows* indicate the 65-kDa ER_α and 61-kDa $ER_\alpha\Delta3$. The $ER_\alpha\Delta3$-mRNA as a percent of total ER was as follows: clone $ER_\alpha\Delta3$–1, 59%; 2, 57%; 3, 64%; 4, 67%. The protein was as follows: $ER_\alpha\Delta3$–1, 36%; 2, 40%; 3, 76%; 4, 60%. (A second determination of the $ER_\alpha\Delta3$-mRNA and protein fraction in clone 1 yielded 66% and 42%, respectively; in clone 4, 67% in both assays).

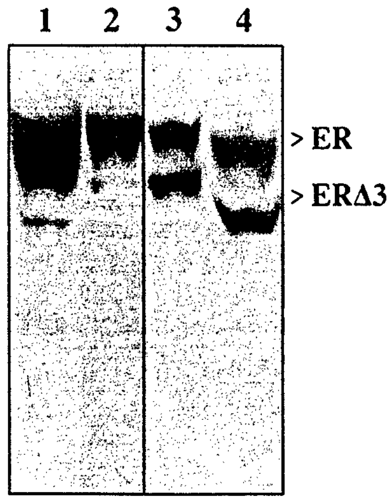

Fig. 4. Identification of the Native 61-kDa Protein as $ER_{\alpha}\Delta3$ on the Basis of Its Dephosphorylation Pattern

Two equal aliquots of ER_{α} immunoprecipitated with rabbit polyclonal anti-ER_{α} antibody (Zymed) from 2 mg of protein extracts of pMV7pool vector control or $ER_{\alpha}\Delta3$–3 clone grown in medium with FBS (to enhance the $ER_{\alpha}\Delta3$ to ER_{α} ratio) were resuspended in 25 μl phosphatase buffer with protease inhibitors (100 μg/ml leupeptin, 100 μg/ml aprotinin, 20 μg/ml pepstatin) and incubated for 30 min at 30 C with 3 U (or without, controls) of CIP (Boehringer Mannheim). The products were analyzed by Western blotting using H226 antibody. The amount of protein loaded per lane was 2.5 times more than in Figs. 3 or 6. Lanes 1 and 2, pMV7pool vector control: lane 1, CIP treatment; lane 2, buffer control; lanes 3 and 4, $ER_{\alpha}\Delta3$–3, lane 3, buffer control; lane 4, CIP treatment. The dephosphorylated shifted doublets of ER_{α} and $ER_{\alpha}\Delta3$ are indicated.

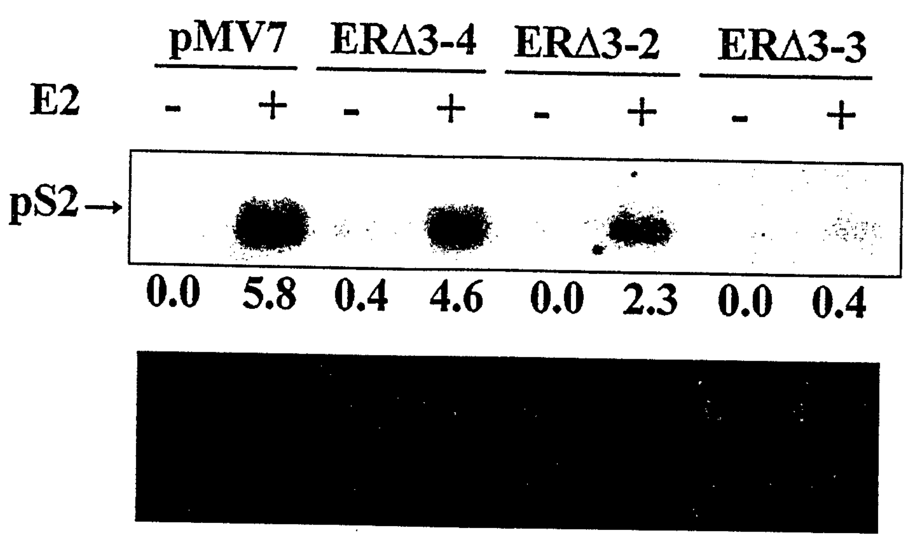

Fig. 5. Effect of $ER_{\alpha}\Delta3$ Expression on Estrogen Regulation of pS2-mRNA

pMV7 pool vector control (1×10^6) and $ER_{\alpha}\Delta3$–3 clonal cells were plated in 100-mm tissue culture dishes in the presence of FBS for 3 days and treated for 48 h either with the pure antiestrogen ICI 164,384 (1×10^{-7} M), to establish the baseline of pS2 expression, or E_2 (1×10^{-8} M). pS2 expression was determined by Northern blot analysis of 20 μg total RNA using pS2 cDNA probe. Ethidium bromide-stained ribosomal RNA (*lower panel*) was used as a loading control. pS2 mRNA in pMV7 pool, and clone $ER_{\alpha}\Delta3$–4, -2, and -3 cells treated either with ICI 164,384 (shown in lanes designated as −) or E_2, (shown in lanes designated + to indicate E_2 addition). Exposure 2 h. Clone ERΔ3–3 expresses the highest level (76% of total) of ERαΔ3 protein (see Fig. 3).

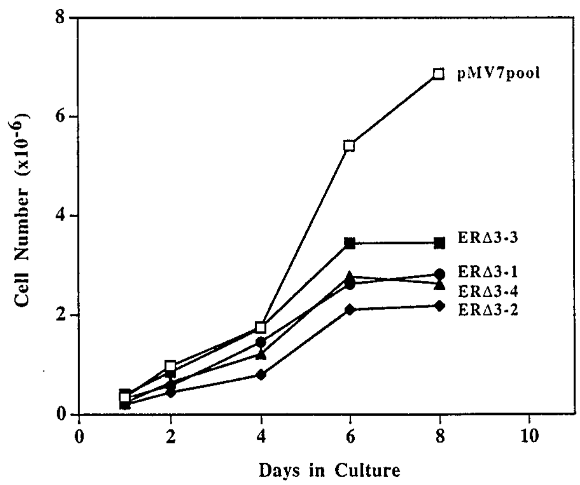

Fig. 6. Effect of $ER_{\alpha}\Delta3$ Expression on Growth Rate and Saturation Density

pMV7pool vector control (0.2×10^6) and $ER_{\alpha}\Delta3$–1, -2, -3, and -4 clonal cells were plated in 60-mm tissue culture dishes in the presence of FBS. Cells were maintained for 8 days and medium was replaced every 3 days. On days 1 and 2 and every second day thereafter, cells in three dishes of each cell type were detached and counted. The results shown are the mean of three determinations. (SEM values were smaller than the symbols and thus are not shown). Comparison of the saturation plateau of each of the four clones and the vector control by ANOVA statistics performed on day 8 showed a significant difference ($P < 0.0001$).

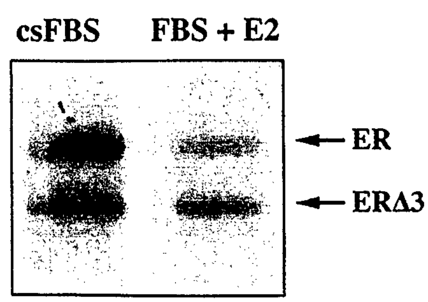

Fig. 7. The Effect of E_2 on the Relative $ER_{\alpha}\Delta3$ to ER_{α} Protein Level

Protein (400 μg), extracted from $ER_{\alpha}\Delta3$–2 cells plated at 4×10^6 per 100-mm dish and grown either in csFBS or FBS with 1×10^{-8} M E_2 for 72 h, was immunoprecipitated with rabbit anti-ER_{α} antibodies (Zymed) and analyzed by Western blotting using H226 antibody as described.

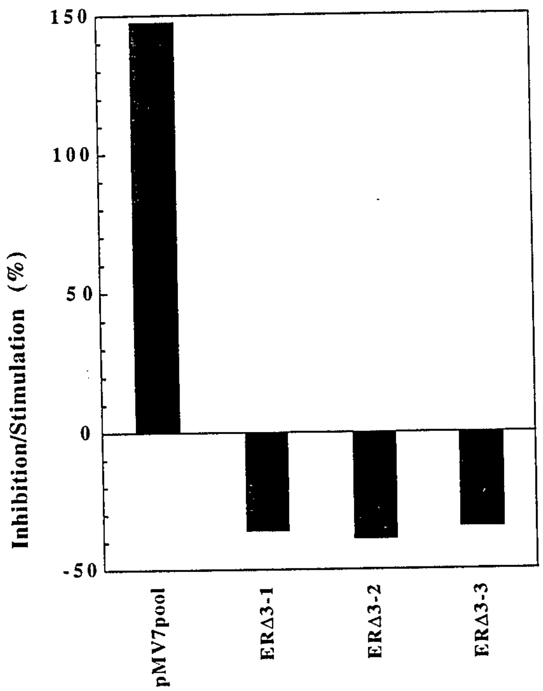

Fig. 8. Anchorage-Independent Growth of $ER_\alpha\Delta3$ and pMV7pool Cells

Low melt agarose (Seaplaque, 1% in lower and 0.4% in upper layer) was prepared in DMEM with insulin (5 μg/ml) and 10% FBS ($\pm 1 \times 10^{-8}$ M E_2). To assess anchorage-independent growth, pMV7pool and $ER_\alpha\Delta3$ clones 1, 2, and 3 cells (2×10^3 cells/ml) mixed with agarose (upper layer) were distributed on top of 5 ml of lower DMEM/agarose layer, grown for 2 weeks, and scored for colony formation. Colonies were scored in one fourth to one half of each dish. The results are the mean of duplicate determinations. Stimulation or inhibition by E_2 is expressed as percent of colonies in agarose containing medium with FBS alone. The cloning efficiency of the pMV7 pool cells under control conditions (medium with FBS alone) was 6.5%.

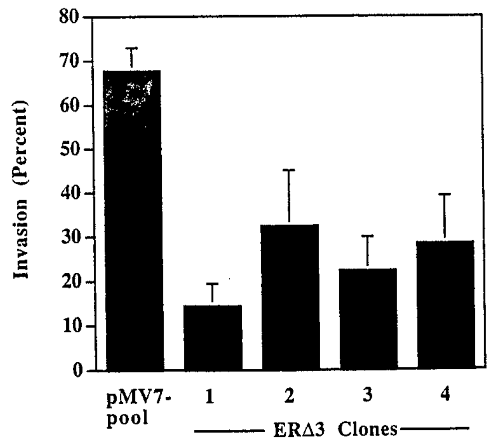

Fig. 9. The Effect of $ER_\alpha\Delta3$ Expression on *in Vivo* Invasion

Eight replicate chick embryo chorioallantoic membranes (CAMs) were inoculated with 3×10^5 cells per CAM of pMV7pool vector control or $ER_\alpha\Delta3$- clones 1, 2, 3, or 4 cells grown in the presence of 1×10^{-8} M E_2 for 72 h and labeled with 0.2 μCi/ml of[^{125}I]UdR for the last 24 h (specific activity 0.1 to 0.2 cpm/cell). Preparation of CAMs for inoculation and quantification of invasion was as described except that CAMS were resealed before inoculation for 22 h. The results are expressed as median percent invasion. Statistical analysis (ANOVA) performed on all pMV7 controls (n = 24) and all $ER_\alpha\Delta3$-expressing clones (n = 32) indicated that the groups were significantly different (P = 0.007).

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